# PHYSIOCHEMICAL ANALYSIS, *IN VIVO* STUDY OF NATURAL FOOD COLOURS AND DEVELOPMENT OF FOOD COLOUR SENSOR

**By**

**J. Yoshia Leela**

**(Reg. No. 21PHFDF001)**

**Full Time Ph. D (without M. Phil.)**

***Supervisor***

**Dr. PL. Sridevi Sivakami**

**A Synopsis Submitted to**

**Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore – 641 043**

**In Partial Fulfilment of the Requirement for the Degree of Doctor of Philosophy in Food Service Management and Dietetics**

**August 2024**

**ABSTRACT**

This study had focused on the impacts of synthetic food colourants through a survey conducted among the food vendors (N=100) and home makers(N=100)of Coimbatore city, Tamilnadu, along with a survey conducted in supermarkets and shops (N=150)for popular brands of synthetic food colourants.Natural food colouring sourcesselected were annatto seeds (*Bixa Orellana*), eucalyptus bark *(Eucalyptus grandis),* madder roots (*Rubia cordifolia*), roselle petals (*Hibiscus sabdariffa*) and tamarind seeds (*Tamarindus indica*). Selected forest plant sources were collected from Forest College and Research Institute, Mettupalayam, Tamilnadu, the forest plant sources were subjected to the processes of aqueous extraction and powdering. Tamarind seeds did not give any colour and was ruled out from the study for further analysis . The shelf life of the aqueous extracts were monitored at room temperature and refrigeration, where a slimy layer was found at the bottom of the glass bottles after a fortnight's time which were confirmed to be microbial growth. Microbial assay for fungal and bacterial identification of species were carried out.Primary toxicity study with brine shrimp assay showed that roselle petals had higher toxicity and was ruled out from further analysis. Chemical analysis phytonutrients were carried out in the rest of the samples which exhibited less antioxidant properties and more presence of phytonutrients. The aqueous extracts were unstable and started to degenerate the colours, thus further processing was done with powdered substances of the selected natural food colourants. Secondary toxicity study of heavy metals using ICPMS was conducted to detect the toxics harmful to human health, in which madder root powder had high amount of lead (3.28560 µg), cadmium (0.05162 µg) and chromium(5.48116 µg), thus, it was ruled out. Other characterization properties were analysed through FESEM, XRD, TGA and photosensitivity. Annatto seeds powder was amorphous in nature with less loss of mass, whereas eucalyptus bark powder was crystalline in nature and had a heavy loss of mass whenever there was increase in temperature. The powdered substances of annatto seeds and eucalyptus bark were optimized through an eight weeks *in vivo* study, which revealed that annatto seeds powder can be consumed 3 mg/kg of body weight (Human Equivalent Dosage , 7 mg) and eucalyptus bark powder of 6 mg/kg of body weight (Human Equivalent Dosage, 15 mg). Later, the animals were sacrificed, their vital organs (kidney, brain, heart, stomach, liver, intestine and spleen) were harvested for histology and hematological parameters. No significant changes were found in the vital organs and blood parameters. Thus, the selected natural food colourants were safe for consumption and was incorporated into selected recipes. Then the recipes were checked for intensity of colours and were organoleptically evaluated. Keeping this in mind, a food colour sensor was fabricated to detect the toxicity in the food colours through permissible limit prescribed by the FSSAI(2021) for red and yellow colours. Fabricated food colour sensor was trained with the data set, to detect the level of toxicity present in the solid foods added with commercial food colourants.

## INTRODUCTION

Foods that attract consumers of all age groups are being added with synthetic food colourants that are stable, cost efficient and harmful for human safety. These food colourants are used in more than the limitations prescribed by Food Safety and Standards Authority of India (FSSAI). Relevant, alternatives for these synthetic food colourants are present in natural form. Thus, in this study the natural form of colourants are explored for bringing in a solution for the greatest uprising demand for food colourants in the food industries.

Around 490 food samples and 62 samples of natural, herbal colours were collected by the Society of Pollution and Environmental Conservation Scientists (SPECS) from various places of India were adulterated with toxic chemical synthetic dyes (*Tribune, 2016*). One of the world's most demanded spices and food colorant saffron was found to be adulterated with sudan dyes (*Petrakis et al. 2017*). Turmeric labelled as natural and organic was highly adulterated with lead chromate (*Erasmus et al. 2021*). Many "organic" labelled foodstuffs like tea powder, jaggery and edible oils were also reported to contain non-permitted colorants (*Pradeshi, 2019*).

The unrestrained use of colorants in Indian street food markets urgently needs to be appraised. Analysis studies on street food vendors in Chennai underpin that 94 percentage are unaware of even the hygiene practices and 74 percentage believe that applying for a license is an arduous task (*Abraham et al., 2017*).FSSAI had published the list of natural and synthetic colorants with permitted limits in two regulations. The non-permitted colours are Fast red, Rhodamine B, Metanil yellow, Bromo-cresol purple, Green S, Sudan 1, 2, 3 and 4, as well as the overuse of permitted colours. There is no distinction between natural colours and the synthetically produced natural colours. As per FSSAI, food products are sold under the label 'contains permitted natural colours/permitted synthetic colours/contains permitted natural and synthetic colours' without any depiction of the used colorant (*FSSAI, 2011*).

Thus, in consideration towards the growing demands the purpose of the study is to have food safety, to ease the need for the hour by developing alternative natural food colourants.To select natural food colourants from plant sources and to analyse their physical, chemical and characterization properties.To measure the shelf-life, analyze the microbial growth, to test the toxicity of the natural food colourants using primary, secondary and *in vivo* model through histopathological and haematological parameters. To incorporate and evaluate the developed natural

food colourants in selected recipes through organoleptic evaluation.To develop a sensor detecting the toxicity in the foods colourants in selected recipes in consideration with the guidelines of FSSAI.

## Need for the Study

1. Common ill-effects in consuming synthetic colourantshave negative impact on cognative development, functioning of the liver and other vital organs.
2. Optimizing the natural food colourantsas alternative for synthetic food colourants for safe human consumption.

## Objective of the Study

***Primary Objective***

To alternate synthetic and artificial food colours as the non-natural colours cause illness to human beings.

***Secondary Objectives***

- To select natural food colourants from plant sources and to analyse their physical, chemical and characterization properties.
- To measure the shelf-life, analyse the microbial growth, to test the toxicity of the natural food colourants using primary, secondary and *in vivo* model through histopathological and haematological parameters

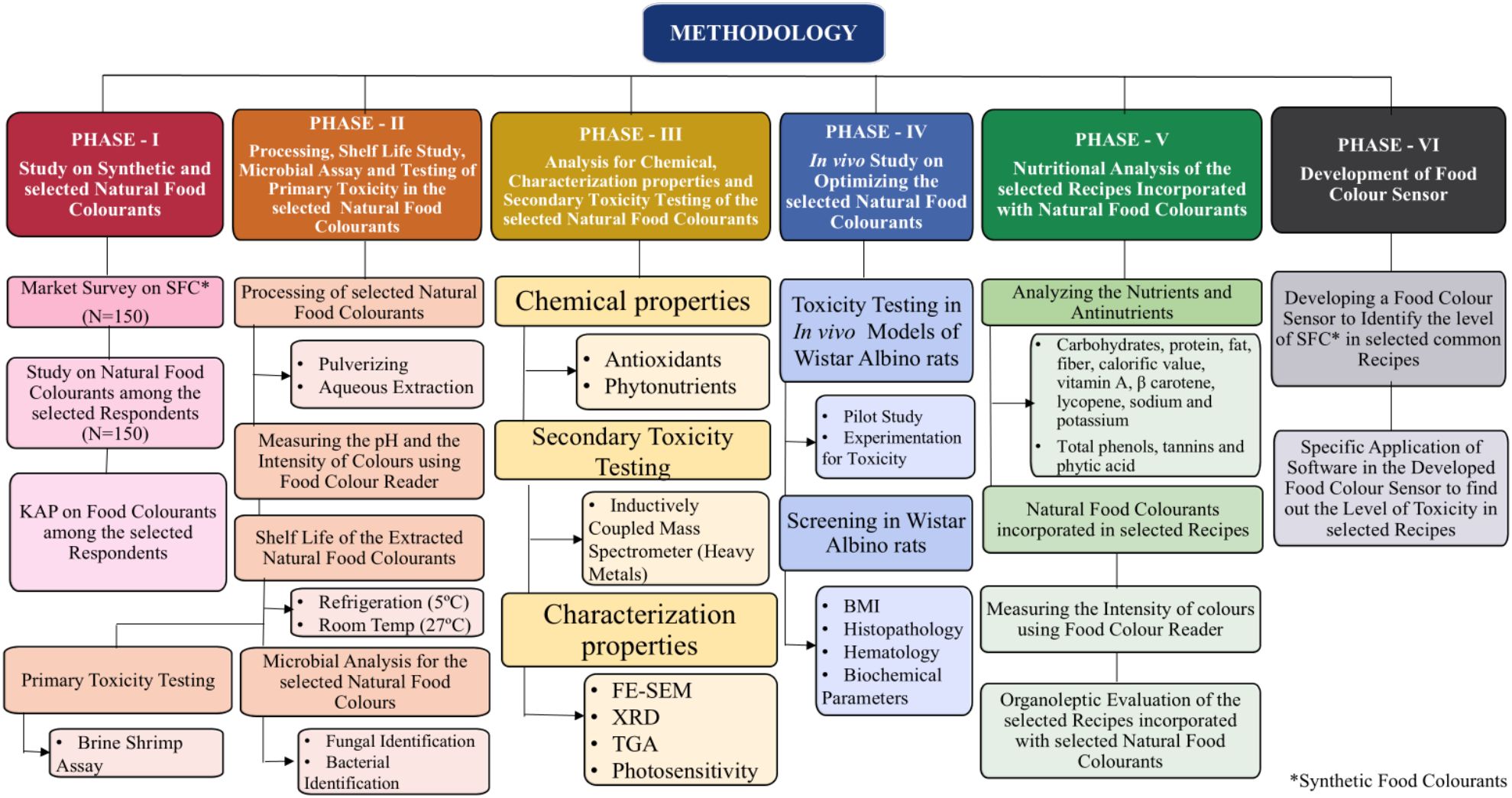
METHODOLOGY
PHASE - I
Study on Synthetic and selected Natural Food Colourants
Market Survey on SFC* (N=150)
Study on Natural Food Colourants among the selected Respondents (N=150)
KAP on Food Colourants among the selected Respondents
Primary Toxicity Testing
Brine Shrimp Assay
PHASE - II
Processing, Shelf Life Study, Microbial Assay and Testing of Primary Toxicity in the selected Natural Food Colourants
Processing of selected Natural Food Colourants
Pulverizing
Aqueous Extraction
Measuring the pH and the Intensity of Colours using Food Colour Reader
Shelf Life of the Extracted Natural Food Colourants
Refrigeration (5ºC)
Room Temp (27ºC)
Microbial Analysis for the selected Natural Food Colours
Fungal Identification
Bacterial Identification
PHASE - III
Analysis for Chemical, Characterization properties and Secondary Toxicity Testing of the selected Natural Food Colourants
Chemical properties
Antioxidants
Phytonutrients
Secondary Toxicity Testing
Inductively Coupled Mass Spectrometer (Heavy Metals)
Characterization properties
FE-SEM
XRD
TGA
Photosensitivity
PHASE - IV
In vivo Study on Optimizing the selected Natural Food Colourants
Toxicity Testing in In vivo Models of Wistar Albino rats
Pilot Study
Experimentation for Toxicity
Screening in Wistar Albino rats
BMI
Histopathology
Hematology
Biochemical Parameters
PHASE - V
Nutritional Analysis of the selected Recipes Incorporated with Natural Food Colourants
Analyzing the Nutrients and Antinutrients
Carbohydrates, protein, fat, fiber, calorific value, vitamin A, β carotene, lycopene, sodium and potassium
Total phenols, tannins and phytic acid
Natural Food Colourants incorporated in selected Recipes
Measuring the Intensity of colours using Food Colour Reader
Organoleptic Evaluation of the selected Recipes incorporated with selected Natural Food Colourants
PHASE - VI
Development of Food Colour Sensor
Developing a Food Colour Sensor to Identify the level of SFC* in selected common Recipes
Specific Application of Software in the Developed Food Colour Sensor to find out the Level of Toxicity in selected Recipes
*Synthetic Food Colourants

**FIGURE – 1**

**METHODOLOGY OF THE STUDY**

**METHODOLOGY –** The methodology of the study is conducted under the following procedures:

**PHASE – I: Study on Synthetic and Natural Food Colourants**

- As an initiation of the study, a detailed survey was conducted on the availability and frequency of usage of synthetic food colourants. In consideration with the frequency in usage of synthetic food colourants, equalized natural colouring sources were selected and analysed.
- This study was conducted on human preferences has obtained the Institutional Human Ethical Clearance from the Institutional Human Ethics Committee with the proposal No. IHEC/22-23/FSMD-1 issued in the year 2023, with the Approval number for the same as AUW/IHEC/FSMD-22-23/XPD-1.
- There were 32 different brands available, in which three brands were very familiar and were sold in higher quantity. The brands' names are coded for confidentiality purpose and the brands are:BK, BU and GC.To alternate these commercial food colours, different plants were studied for extracting natural food colours, considering their availability and their coloured pigments.
- The coloured pigments present in natural form were narrowed down in consideration with their local availability, where the plant based sources were selected for further extraction process after a survey conducted among the selected home makers as respondents.
- The natural food colourant samples of six different plant origins were selected, namely: annatto seeds (*Bixa orellana*) that produces orange red colour, eucalyptus bark (*Eucalyptus grandis*) that gives brown colour, madder roots (*Rubia cordifolia*) that produces brown red colour, tanner's cassia (*Senna auriculata*) with yellow orange colour, roselle petals (*Hibiscus sabdariffa*) that gives dark red colour and tamarind seeds (*Tamarindus indica*) were colourless.
- The selected natural food colourants were studies in detail, bringing to light their various medicinal properties and different remedial therapeutic aspects.
- Using a well structured interview schedule, selected respondents of home makers (N=100) and food vendors (N=100), their knowledge, attitude and practice in consideration through the foods that they prepare, the colours they use, the consumer preferred colours along with the frequency of usage of type of foods were analysed.

**PHASE – II: Processing, Shelf Life Study, Microbial Assay and Testing of Primary Toxicity in the Selected Natural Food Colourants**

- For the process of extraction of colours, the naturally available plant sources that produce colours, which can be partially relevant to that of the synthetic colours commercially available in the market were selected.

- The selected forest plant sources were collected from Forest College and Research Institute, Mettupalayam, Tamilnadu, the substances were subjected to the processes of aqueous extraction, stored in glass bottles and powdering, where the fine powdered particles were packed in small zip lock bags.
- The selected plant based sources were boiled in water bath at 60ºC for 60 minutes, cooled, filtered into sterile glass bottles and stored at room temperature and refrigerator to study the shelf life.
- There was in change in intensity of colours in the aqueous extracts and so they were measured using Food Colour Reader and the pH of the extracts extracted by boiling extraction method was measured using pH meter.
- Quick contamination was found within a period of 10 days. On the $10^{th}$ day, the natural food colourants extracts stored in the room temperature started forming creamy layers. Same way on the $15^{th}$ day, the extracts placed in the refrigerator (5°C), formed a slimy thick layer at the bottom of the bottle and the colour of the extract was too dark than the original colour, confirmed to be microbial growth.
- Fungal and bacterial colonies were identified after inoculating it into a sterilized petric plates using standard pour plate culture with Rose Bengal Chloramphenicol Agar Medium and Nutrient Agar Medium, respectively.
- The plates were kept at room temperature (27°C) for the formation of colonies. The number of colonies for fungal identification formed on the third day and for the bacterial identification on the fifth day was counted and recorded.
- The natural food colourants sample were taken in different concentration as 100, 250, 500, 1000 and 1500µl in each beaker containing saline solution. The movement of the 30 shrimpintroduced into the natural food colour samples was monitored at intervals of 1, 2, 4, 6 and 24 hour continuously. There were three groups, in which the shrimp were introduced into blank solution group, control group [Potassium dichromate – $K_2Cr_2O_7$ (1mg/ml)] and prepared samples with different concentrations. The mortality of shrimp was calculated after 24 hour.

**PHASE – III: Analysis for Chemical, Characterization properties and Secondary Toxicity Testing of the Natural Food Colourants**

- The protocol used in evaluating the antioxidant capacity of compounds is 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method through the scavenging capacity of free radicals in the natural colourants, reducing the DPPH radical with ascorbic acid as standard.

- The extraction samples of natural food colourants were screened for detecting the metabolites like alkaloids, flavonoids, sterols, terpenoids, anthraquinone, anthocyanin, proteins, phenolic compounds, quinones, carbohydrates, tannin, saponines, cardiac glycosides, glycoside's test, lignin, coumarins and volatile oils respectively, as they are preliminary phytochemicals.
- Inductively Coupled Plasma Mass Spectrometer couples under the use of an ICP with MS for elemental analysis in natural food colourants along with the synthetic food colourants by generation of ions was carried out to detect the presence of toxic heavy metals that are harmful for consumption.Field Emission Scanning Electron Microscope (FE-SEM) is an advanced technology used in capturing the microstructure image of any material, to identify the morphological structure of the materials.
- X-ray Diffraction was used to determine the composition of crystallites present in the natural food colourants using monochromatic x-ray beams.Thermal Gravimetric Analysis is a technique, where the sample is heated to a constant weight at each of a series of increasing temperature, subjected to continuous increase in temperature at a constant heating rate, that is usually linear with time.
- Photosensitivity is the illumination condition monitored for degradation process in the solution samples, measuredusing UV spectrophotometer.

**PHASE – IV: *In vivo* Study**

- The in vivo studied carried out to test the toxicity has obtained the permit from the Institutional Animal Ethical Committee with the approval number of AIW:IAEC.2023:03 for the species of wistar albinao rats for a period of 12 months on 14/03/2023.
- Adult wistar albino rats which weighed about 200-250g of 8-12 weeks of age were selected, inhabited to house under the standard environmental condition of 25±2°C with sufficient light and dark cycles of 12 hours each.
- According to Organization for Economic Cooperation and Development Guidelines *(2018*), a pilot study was done in which aqueous solution of 2ml/100g of body weight, where the natural food colourants were orally fed to the rats to optimize the level of threshold in ppm, fed continuously for five days and two days of wash out period by increasing the dosage every week.
- During an eight week study for experimentation, the animals' meal pattern and weight were checked every week and on the $57^{th}$ day the animals were harvested, their organs and blood were taken for histopathological and haematological studies. Later, Human Equivalent Dosage was also calculated using *Anroop B. Nair, et al., (2016)* formula,

**PHASE – V: Nutrient and Organoleptic Analysis of the Selected Recipes Incorporated with Natural Food Colourants**

- The major nutrients like carbohydrates, protein, fat, fiber along with the minor nutrients like the vitamins and minerals were analysed for 100 g of the natural food colourants using AOAC method.
- Traditional and major consumed recipes such as kesari, motichoorladoo, jalebi, chilli gobi, chilli chicken, aloo bajji, aloo bonda, tutti frutti, apple jam and jelly are selected for incorporation of natural colourants in different variations.
- Ten trained panel members evaluated the food products using five hedonic scale rating in individual score cards.

**PHASE – VI: Development of Food Colour Sensor**

- The color sensor TCS34725 is used for the development of sensor in detecting colours, light-to-digital converter that provides red, green, blue(RGB ) and clear light sensing data digitally via the use of an IR filter and an I2Cinterface.The surface is illuminated with a white light source.
- In order to measure the RGB color wavelengths, the sensor then activates three filters, each of which has a wavelength sensitivity of three. These three hues are used to determine the color of the material.
- Thonny is a Python programming language Integrated Development Environment(IDE).As for the regulations of Food Safety and Standards Authority of India (FSSAI), the toxicity level and the permissible level of usage of synthetic food colourants were added to the data set of the sensor and trained.
- Productive utilization of the developed signal processing system can aid the community in analyzing the toxicity in the food colourants that are being used in selected foods.

## FINDINGS OF THE STUDY

**PHASE – I: Study on Synthetic and Natural Food Colourants**

- From the market survey, the researcher had conducted the study on the highly used commercial brands available in the local supermarkets and shops. The brands preferred were BK, BU and GC which were sold in 28, 21 and 34 supermarkets respectively.
- The present study revealed that 78 percent of the home makers were familiar with all the natural colours except for annatto seeds (*Bixa orellana*) only 18 percent of the home makers were aware of annatto seeds come under natural food colourants.
- Likewise, the knowledge on eucalyptus bark (*Eucalyptus grandis*), madder roots (*Rubia cordifolia*), roselle petals (*Hibiscus sabdariffa*), tamarind seeds (*Tamarindus indica*) and tanner’s

cassia petals (*Senna auriculata*) were accounting to only 23 percent, 18 percent, 19 percent, 21 percent and 16 percent respectively.

- From this survey conducted among the home makers and food vendors in Coimbatore, its evident that, 81 percentage of the selected respondents had knowledge on food colourants, where 75 percentage of them had the tendency and good attitude towards the rules and regulations formulated by FSSAI. But, according to their style of preparation, target customers and commercial competition in the society. All the selected respondents had the practice of using synthetic food colourants in the food that they prepare on day-to-day basis.
- The food vendors had also reasoned out their usage of synthetic food colourants in the food they prepare, explaining that the food colourants are added to the food to attract either a specific target group of population or to have more customers than their competitors, especially to make the food look more appetizing, attractive and appealing to consume.

**PHASE – II: Processing, Shelf Life Study, Microbial Assay and Testing of Primary Toxicity in the selected Natural Food Colourants**

- The intensity of the colour values also proved that the boiled extracts had shown discolouration for every two days with less stability, whereas the powdered substances did not show any change in colour for testing the physical characteristics.
- The compounds were analyzed for pH ranges to analysis acidic in nature, where eucalyptus bark (*Eucalyptus grandis)* and roselle petals (*Hibiscus sabdariffa*) extracts were measured to be 4.57 mol/L and 3.43 mol/L of base with a yield of 160 ml and 180 ml from 250 ml. Whereas madder root (*Rubia cordifolia*) extract was slightly acidic with 5.69 mol/L with an yield 0f 170 ml and annatto seeds (*Bixa orellana*) extract had very mild acidity of 6.33 mol/L with an yield of 230 ml.
- Through the physical characteristics of the fungal group the species were identified manually. White gray layer was *Rhizopus*, greenish black spongy layer was *Aspergillus fumigatus*, pale green root like layer was found to be *Trichoderma* and olive green spots were found to be *Pencilium*. All these four species were found in the extracts of annatto seeds (*Bixa orellana*).
- Whereas in eucalyptus bark extracts (*Eucalyptus grandis*) showed three colonies were identified with green strands of hairy cotton cluster was formed, it was identified to be *Aspergillus niger*, white hairy layer with spores was a species of mucor and shiny velvet-black fluffy growth was *Curvularia* species.
- Two different fungal colonies were observed in madder roots (*Rubia cordifolia*) extract. The fungal colonies of *Flavus* which was yellow white spongy spores and *Rhizopus stolonifer* which was a white fluffy layer were observed.

- In rosella petals extract (*Hibiscus sabdariffa*), green strands of hairy cotton clusters known as *Aspergillus niger*, yellow green spongy spores known as flavus and olive green spots known as *Penciliu*mwas identified.
- The major bacterial colonies foundthrough Crystal-Violet Gram Staining Method, are: madder roots (*Rubia cordifolia*) showed the presence of *Bacillus* species, eucalyptus bark (*Eucalyptus grandis*) showed the presence of *Staphylococcus* species, annatto seeds (*Bixa orellana*) showed the presence of *Bacillus* species and roselle petals (*Hibiscus sabdariffa*) showed the presence of *Staphylococcus* species.
- The brine shrimp lethality assay carried out for powdered substances show less mortality rate and for further analysis, the powdered form of natural food colourants was used for further analysis.Whereas, in roselle petals (*Hibiscus sabdariffa*) all the shrimp were found dead at different concentration, had proved to be toxic in nature so it was ruled out from the study for further analysis.

**PHASE – III: Analysis for Chemical, Characterization properties and Secondary Toxicity Testing of the Natural Food Colourants**

- Analysis for antioxidants was carried out, in the annatto seeds (*Bixa orellana*), the percentage of inhibition in aqueous extracts have been increased as that of the standard ascorbic solution from 57.39 percentage to 92.17 percentage with increase in concentration from 10µl to 500µl respectively, whereas in the powdered substances the rate of inhibition has decreased from 82.79 percentage to 54.92 percentage. In the eucalyptus barks (*E grandis*) extract, the percentage of inhibition was decreased from 97.39 percentage to 68.70 percentage that is from 10 µl to 750 µl respectively. In eucalyptus barks powder there was decrease in inhibition percentage from 67.21 percentage to 54.10 percentage.
- In the test for the phytochemicals, the presence of 17 different metabolites was tested. The madder roots' (*Rubia cordifolia*) aqueous extracts had the presence of six metabolites and in powdered substances, there were seven metabolites out of 17 metabolites. While, testing the eucalyptus bark (*Eucalyptus grandis*), there were nine and six metabolites, out of 17 metabolites in aqueous extracts and powdered substances respectively. As for annatto seeds, the aqueous extracts and powdered substances had an equal count of six metabolites each out of 17 metabolites.
- Then secondary toxicity study was carried out through Inductively coupled plasma mass spectrometer (ICPMS), through which heavy metals were analyzed. In this method of detecting toxins, madder roots (*Rubia cordifolia*) proved to have larger amount of lead 3.28560 µg,

cadmium 0.05162 µg, chromium 5.48116 µg and mercury 0.15526 µgin its natural form. Thus, madder roots (*Rubia cordifolia*) was not suggested for further studies.

- In the FESEM of eucalyptus bark (*Eucalyptus grandis*) powder, the needle like structure exhibits the presence of residue of cell lignin particles that are crystalline cellulose embedded in the form of lignin matrix responsible for the rigidity of the bark substance.
- From the XRD, the higher peak intensity of the annatto seeds powder have shown that there are higher number of atoms in the crystal proving it to be amorphous is nature, whereas the highest peak in the eucalyptus bark powder indicated that the higher degree of crystallinity is due to the large crystals of particles present in it, proving it to be crystalline in nature.
- In TGA, the loss of mass has taken place before 200ºC in both the natural food colourants of annatto seeds and eucalyptus bark, which proves that they are temperature resistant as all forms and types of cooking takes place between 60 ºC -200ºC, respectively.
- During photosensitive analysis, from zero hour to twenty four hours, the degradation in concentration was 0.32 µg loss of strength in the concentration of annatto seeds (*Bixa orellana*), whereas the same level of degradation was measured as 0.32 µg of loss of concentration due to degradation is found in eucalyptus bark (*Eucalyptus grandis*). There is degeneration of colours when exposed to light for a longer period of time.

**PHASE – IV: *In vivo* Study**

- The animals were divided into groups with five animals in each experimental group and two animals in control group. Group – 1 is the experimental group of eucalyptus barks (*Eucalyptus grandis)*with 6 mg and Group – II is the experimental group of annatto seeds (*Bixa orellana*) with 3 mg, the Wistar Albino Rats wereorally fed for 56 days with the optimized dosage, continuously.
- The BMI of the wistar albino rats before and after the experimentation was calculated by measuring its weight. The paired sample t-test was applied to find the statistical difference before and after experimentation. The p value was greater than 0.05, which signifies that there is no significant difference before and experimentation, proving it to be that there has been no change in the weight of the body mass due to constant consumption of water or either due to the natural food colourants.
- After 56 days, the animals were euthanasia for histopathology study, to identify the effects of the natural food colourant in both the experimental groups. All the animals were harvested and the organs (the heart, liver, kidney, intestine, stomach and spleen) were collected in solution of formaldehyde with water in the ratio of 1:10.

- No pathologies were recorded in the histological sections of the vital organs (brain, liver, kidney, heart, stomach, intestine and spleen) of the control group. Equally, there was no change in the creatinine after administration of eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) powder extracts when compared to the control group.Thus, this study proves that the colourants optimized are nontoxic in nature and can be used as natural food colourant.
- The measured mean values of the hematological and biochemical parameters have their ranges between the reference range, which indicates that there are no biochemical changes that has been taken place in the experimental group of rats of eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) that were continuous fed for 56 days of sub chronic period.
- The Human Equivalent Dosage (HED) was calculated using the animal dosage level *(Anroop B. Nair, et al., 2016)* formula, where total dosage of annatto seeds (*Bixa Orellana*) for 60 kg human is 7 mg/day and total dosage of eucalyptus bark (*Eucalyptus grandis*) for 60 kg human is 15 mg/day.

**PHASE – V: Nutrient and Organoleptic Analysis of the Selected Recipes Incorporated with Natural Food Colourants**

- The nutrients analyzed for protein content in the annatto seeds (*Bixa orellana*) andeucalyptus bark (*Eucalyptus grandis*) powders were 5.92 g and 2.13 g, respectively, whereas fat content was of 5.92 g and 2.13g, crude fiber was 16.84 g and 15.14 g, carbohydrates was 77.12 g and 83.39 g and the calorific value was 351.33 kcal and 345.59 kcal respectively.
- The micronutrients analysed in annatto seeds (*Bixa orellana*) andeucalyptus bark (*Eucalyptus grandis*) powders were Vitamin A with 175.13 and 75.51 IU, respectively, beta carotene of 105076.39 mg and 45308.27 mg and lycopene of 28.09 and 34.69 mg respectively. The minerals analysed for sodium was 7.42 µg and 1.66 µg, and potassium was 609.28 and 91.03 µg, respectively in annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) powders.
- The recipes prepared and incorporated with the natural food colourants are as follows: kesari, motichoor ladoo, chilli gobi, chilli chicken, tutti frutti, jalebi, aloo bajji, aloo bonda, apple jam and jelly. Using the standard procedure, these recipes were prepared and their intensity in colour was also recorded. In the standard preparation of all the recipes the highest popular and most commonly (through the survey) used synthetic food colourant BU was used to note the colour difference between the natural and the synthetic food colourants.
- The common recipe of kesari, in comparison to the standard recipe prepared with BU, annatto seeds (*Bixa orellana*) powder of variation – III had bright hue of 27.23, when compared with eucalyptus bark (*Eucalyptus grandis*) of hue 24.69. In comparison to the standard recipe of ladoo,

the variations – I and II of annatto seeds (*Bixa orellana*) with 22.57 and 26.56 and variation – III of eucalyptus bark (*Eucalyptus grandis*) 21.16 hue had their hues equal vent to each other.

- The variations had their increase in hue as the amount of natural food colourants were increased by 1mg each in each recipe, where the standard procedure was followed. Variations I, II and III of annatto seeds (*Bixa orellana*) powder had 15.15, 19.44 and 26.18 hue respectively. The variation I, II and III added with eucalyptus bark powder (*Eucalyptus grandis*) and the colour intensity of the recipes increased as 16.91, 22.72 and 23.70 hue, respectively.The standard recipe and the variation – II of annatto seeds (*Bixa* orellana) powder incorporated recipe of aloo bajji had the identical colour and appearance. The variations – I, II and III of eucalyptus bark powder (*Eucalyptus grandis*), the range of intensity of colour was 50.95, 51.29 and 51.44 hue. The standard recipe aloo banda had its hue value of 52.04, whereas in the variation – I, II and III, the hue has increased from 51.20 to 51.43 and 52.39 respectively. Likewise, in the variation – I, II and III of eucalyptus bark (*Eucalyptus grandis*) also there is an increase in hue from 49.73, 51.07 and 51.62 respectively.
- In the fruit preservatives of tutti frutti, the standard food colourant used was BU, which had the hue of 12.51. The variations I, II and III of annatto seeds (*Bixa* orellana) had slight increase in the range of hue from 11.40 to 13.48 and then to 13.87 hue. The variations – I, II and III of eucalyptus bark powder (*Eucalyptus grandis*) had the colour intensity increased from 8.88, 9.55 and 11.66, respectively. In variation of apple jam – I, II and III of annatto seeds (*Bixa* orelaana), the hue has increased from 51.79, 53.14 and 53.36 respectively. Likewise, in the of variations I, II and III of eucalyptus bark (*Eucalyptus grandis*), the hue has increased from 53.17, 53.24 and 53.27, respectively.
- In the sensory evaluation of the products, kesari and motichoor ladoo with variation – II had been evaluated as very good with four points in the hedonic scale rating (4.5±0.8, 4.2±0.6 and 4.6±0.7, 4.1±0.7). Whereas, variation – I of jalebi (3.4±1.0, 3.1±1.0) of both the natural food colourants were preferred by the panellists. In the snacks recipes, variation - I (4.3±0.7, 4.2±0.8) of chilli gobi, variation – II and III of chilli chicken (4.8±0.4, 4.4±0.7), variation – II (4.2±0.9, 3.9±0.9) of aloo bajji and variation – II of annatto seeds (4±0.8) and variation – I (3.4±0.7) of aloo bonda had been favoured by the semi trained panelists. Variation – II of all the fruit preservatives of tutti frutti (annatto seeds – 3.8±0.9 and eucalyptus bark – 3.9±0.6) and apple jam (annatto seeds – 4±1.1 and variation – I of eucalyptus bark – 3.8±0.8), and variation – I of jelly of both the natural food colourants (3.4±0.7 and 3.2±0.6) were highly accepted by the panelists with a very good score through the hedonic scale rating.

**PHASE – VI: Development of Food Colour Sensor**

- The toxicity of food colourantshas paved way for the utilization ofsensorsandwebpages holding a guaranteed effectiveness, exactness and accuracy over the supply chain of data set grouped for specific recipe, eventually profiting and regulating the need for the usage of the device among common people and food vendors to organize their preparation of foods using food colourants.
- From the data set built using the limitations of FSSAI (2011, 2021), the source code is being used to run the program implementing KNN algorithm to detect and execute the values of toxicity.
- The implemented algorithm is used to construct the graph set that is being used to determine the toxicity values in selected foods classifying it as mild, moderate and severe level of toxicity.
- Common commercial recipes were selected and placed in the developed food colour sensor to test its efficacy. Kesari added with orange colourant in the quantity that is used for preparation at home and the level of toxicity was 4.5 mg, when compared to the FSSAI guidelines, it is permissible limit. But when an extra amount was added it showed as toxic, exceeding the permissible level.
- Likewise, chilly chicken prepared using three variations of 1, 2 and 3 has all shown to be safe for consumption with the range of 4.61, 4.64 and 4.84 respectively. Commercially available chilly gobi, jalebi and ladoo added with yellow and orange synthetic food colourants were checked for permissible level for consumption, where the results were 4.20, 4.76, 5.08 and 5.45 mg, respectively.
- To check the efficacy with the natural colourants, vegetable cutlet, mango pickle and tomato powder of natural form were place in the sensor and it showed the results below the permissible limit proving its efficacy.

**SUMMARY AND CONCLUSION**

- Market survey on synthetic food colourants was carried out in 150 supermarkets and shops. Survey was conducted among selected respondents of home makers and food vendors (N=100).A brief study on the selected natural food colourants were carried out.
- Physiochemical analysis (Phytonutrients: Annatto seeds – 6 out of 17 metabolites in both aqueous and powdered form; eucalyptus bark – 9 and 7 out of 17 metabolites; madder root – 6 and 7 metabolites in aqueous and powdered form), characterization and toxicity – Primary toxicity in Brine shrimp Assay, where roselle petals were extremely toxic in nature (1500 µg/ml – 100 percent dead in aqueous and 80 percent dead in powdered form), Secondary toxicity in ICPMS - Madder root (*Rubia cordifolia*) proved to have lead 3.28560 µg, cadmium 0.05162 µg and chromium 5.48116 µg in the highest quantity, than the other selected natural food colourants.

- *In vivo* models were used in optimization of natural food colourants (6 mg of Eucalyptus bark powder, 3 mg of Annatto seeds powder) to be consumed on daily basis.The optimized natural food colourants were incorporated in selected traditional sweets, snacks and fruit preservatives.
- The intensity of their colour was checked and sensory attributes were evaluated organoleptically through ten semi trained panel members.The overall acceptability of the selected recipes were considered and common recipes were selected for the development of AI based food colour toxicity detecting sensor.
- Data set used to train the sensor were the limitations of set by FSSAI (2021). Webpage and a sensor based device were designed.The selected recipes were analyzed for synthetic food colour toxins and the results were recorded using the webpage.
- The study on natural food colourants as alternatives for synthetic food colourants were carried out in a simpler way as the target group in the community are home makers and food vendors.

**LIMITATIONS OF THE STUDY**

- To incorporate the natural food colourants in frozen foods like ice cream, desserts and confectionaries, also in packaging materials.
- To reach out to a wider target groups of food vendors from other food outlets.
- To provide with sufficient number of wistar albino rats for control group.

**RECOMMENDATIONS OF THE STUDY**

- Future study to be conducted on seasonal plants as colourants and to find ways to store them in proper condition.
- To incorporate the natural food colourants in industrial products in various forms like edible cutlery, edible packaging materials, edible ink, etc.
- To develop various forms of natural food colourants, not only powders, but also in liquid forms like emulsions, dessert toppings and syrups.
- To commercialize the food colour sensor that can be trained to detect toxicity in semi solid and liquid foods.

**REFERENCES**


- Abraham S, Krishnan AT (2017) An analysis of how street food in India can be made safe food. Int J Curr Eng Sci Res 4(12):57–64.

- Erasmus WS, van Hasselt L, Ebbinge ML, van Ruth MS (2021) Real or fake yellow in the vibrant colour craze: rapid detection of lead chromate in turmeric. Food Control 12:107714. https://doi.org/10.1016/j.foodcont.2020.107714.
- FSSAI (2009), Food Safety and Standards regulations. https://old.fssai.gov.in/Portals/0/FSSAI%20regulations.pdf.
- FSSAI (2011) Food safety and standards (food products standards and food additives) regulations. https://www.fssai.gov.in/cms/food-safety-and-standards-regulations.php.
- Petrakis AE, Caglini RL, Tarantilis PA, Polissiou GM, Consonni R (2017) Sudan dyes in adulterated saffron (*Crocus sativus* L.): identification and quantification by $^{1}$H NMR. Food Chem 217:418–424. https://doi.org/10.1016/j.foodchem.2016.08.078.
- Pradeshi S (2019) Food adulteration: injurious adulterants and contaminants in foods and their health effects and its safety measures in India. Int J Sci Res 4:229–236.
- The Tribune (2016) Adulteration in Holicolours, fooditems. https://www.tribuneindia.com/news/archive/features/adulteration-in-holicolours-food-items-212822.